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Musculoskeletal Pathology

Dlx5, a Positive Regulator of Osteoblastogenesis, is Essential for Osteoblast-Osteoclast Coupling

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The homeodomain protein Dlx5 is an activator of *Runx2* (a key regulator of osteogenesis) and is thought to be an important regulator of bone formation. At present, however, the perinatal lethality of *Dlx5*-null mice has hampered the elucidation of its function in osteogenesis. Here we provide the first analysis of the effects of *Dlx5* inactivation on bone development. Femurs of *Dlx5*-null mouse embryos at the end of gestation exhibit a reduction in both total and trabecular bone volume associated with increased trabecular separation and reduced trabecular number. These parameters are often associated with pathological conditions characterized by reduced osteoblast activity and increased bone resorption. *Dlx5*$^{-/-}$ osteoblasts in culture display reduced proliferation and differentiation rate and reduction of *Runx2*, *Osx*, *Osteocalcin* and *Bone Sialoprotein* expression. In addition to impaired osteoblast function, *Dlx5*$^{-/-}$ femurs exhibit significant increases in osteoclast number. As *Dlx5* is not expressed by osteoclasts, we suggest that its osteoblastic expression might control osteoblast/osteoclast coupling. Cultured *Dlx5*$^{-/-}$ osteoblasts displayed a higher RANKL/OPG ratio. Furthermore, *Dlx5*$^{-/-}$ osteoblasts induced a higher number of TRAP-positive multinucleated cells in normal spleen cultures with a globally increased resorption activity. These findings suggest that Dlx5 is a central regulator of bone turnover as it activates bone formation directly and bone resorption indirectly. *(Am J Pathol 2008, 173:773–780; DOI: 10.2353/ajpath.2008.080243)*

The process of osteogenesis depends from complex regulatory networks involving molecular signals and transcription factors,[1,2] deciphering their complexity is one of the future challenges in bone biology. Gene deletion studies have allowed the identification of transcription factors required for the specification and/or differentiation of the osteoblastic lineage. These include, for example Runx2, Osx, Atf4, Msx1, Msx2, Twist, AP1(Fos/Jun), Krox20, Sp3, and members of the Dlx homeobox family.[2]

Vertebrate *Dlx* genes are transcription factors that share a highly conserved homeodomain, homologous to that of *Drosophila Distal-less (Dll)*. The mouse and human Dlx gene system is formed by three bigene clusters : *Dlx*−1 and *Dlx*−2; *Dlx*−5 and *Dlx*−6; *Dlx*−3 and *Dlx*−4.[3–5] All Dlx genes might play a role in chondrogenesis and/or osteogenesis.[6] In particular, *Dlx5* is expressed already at very early stages of bone development[7] and has been proposed to play a central role in the control of osteogenesis. Long bones of *Dlx5*$^{-/-}$ mutant mice present a narrower hypertrophic zone[8] and a defective trabecular component.[9] The calvaria of the *Dlx5* mutants display delayed ossification resulting in open fontanellae.[9] Several recent *in vitro* studies have highlighted the fact that *Dlx5* act at multiple stages of chondrogenesis and osteogenesis by controlling the expression of bone-related genes. Co-immunoprecipitation assays have shown complexes containing both *Dlx5* and *Runx2,* a key regulator of osteogenesis[10,11] and multiple *Dlx5* responsive elements have been identified in the Runx2 P1 promotor.[12] Actually, *Dlx5* could be at the same time a downstream target of *Runx2* and an upstream regulator of *Runx2 type II*.[13,14] Many early and late markers of osteoblastic differentiation are potential direct targets of *Dlx5*. Osterix (Osx), a zinc finger transcription factor, is specifically expressed by osteoblasts during bone development, its inactivation leads to a lack of bone mineralization. *Osx* is a direct target of Runx2;[15] its BMP-2-dependent induction is mediated by Dlx5 through a specific homeodomain responsive element.[16,17] Alkaline phosphatase and osteocalcin have

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been reported to be responsive to *Dlx5*.[18,19] *Dlx5* was found to bind directly to the conserved homeobox-binding site (TTAATTA) of bone sialoprotein (BSP) and to stimulate positively its transcription.[20]

Dlx5-null mice die at birth due to a defect in their respiratory system. The difficulty of performing quantitative studies on the bone of newborn mice has so far hampered the direct analysis of the function of this gene *in vivo*. Most of the available information on the involvement of *Dlx5* in bone growth and development derive either from the observation of alizarin-red stained newborn skeletons[9] or from *in vitro* studies not involving mutant cells. In this study we use our *Dlx5*-null model[9] to finally clarify the role of Dlx5 in bone development. Our results demonstrate that $Dlx5^{-/-}$ mice have a significant decrease in bone volume and that their primary osteoblasts have a reduced proliferation and differentiation capacity with a higher potential to induce osteoclastogenesis.

Materials and Methods

Animals

Mice with targeted disruption of *Dlx5* have been previously described.[9] In these mice the first and second exons of *Dlx5* are replaced by the *lacZ* reporter. PCR genotyping and β-gal staining were performed as described.[9]

Histomorphometry

Femurs were prepared from E18.5 embryos and embedded in methylmetacrylate as described.[21] Histomorphometric parameters were measured in accordance to the ASBMR nomenclature[22] on 5 μm sections using a Nikon microscope interfaced with the software package Microvision Instruments (Evry, France). Sections were stained with aniline blue. For TRAP detection, sections were stained with a 50 mmol/L sodium tartrate and naphtol ASTR phosphate (Sigma, St Louis, France). Total bone volume was measured between the two chondro-osseous junctions; all other measurements were taken beginning at a standard point in the femur 100 μm below the growth plate not including the diaphyseal area. In all cases groups of littermates were analyzed.

Immunohistochemistry

Immunohistochemistry was performed in Tris-buffered saline (Tris 50 mmol/L, pH 7.6, NaCl 150 mmol/L), using standard protocols.[23] Rabbit anti-BSP antibody (LF-87) was kindly provided by Dr Larry Fisher (NIH, USA). Rabbit antisera were revealed with goat anti-rabbit (EnVision, Dako) and peroxidase-conjugated secondary antibodies. Peroxidase reaction was performed with DAB (Dako). Deparaffinized sections were incubated overnight at 4°C with the primary BSP antibody. Negative controls included sections without the primary antibody or with irrelevant antibodies.

Primary Cell Cultures

Osteoblasts were isolated from calvariae of 18.5 dpc embryos as described.[24] Cells isolated from the last two digests were pooled and plated in T-25 tissue culture flask in α-modified minimum essential medium (α-MEM) (Invitrogen, France) containing 10% fetal calf serum (FCS) and antibiotic (100 mg/ml of penicillin/streptomycin). After three days of incubation at 37°C, attached cells from each flask were collected by trypsinization (0.05% trypsin), cells of the same genotype were pooled. To measure mineralization, the culture medium was supplemented with ascorbic acid (50μmol/L) and Na-β-glycerophosphate (10mmol/L), for other assays only with ascorbic acid.

Cell Proliferation, Differentiation and Mineralization Assay

Cultures were pulsed for 18 hours with BrdU in the same medium as above supplemented with 1% FCS; BrdU incorporation was determined using the Cell Proliferation Elisa kit (GE Health care, Burkinghamshire, UK). At each time point, measurements were performed on three wells for each genotype and averaged.

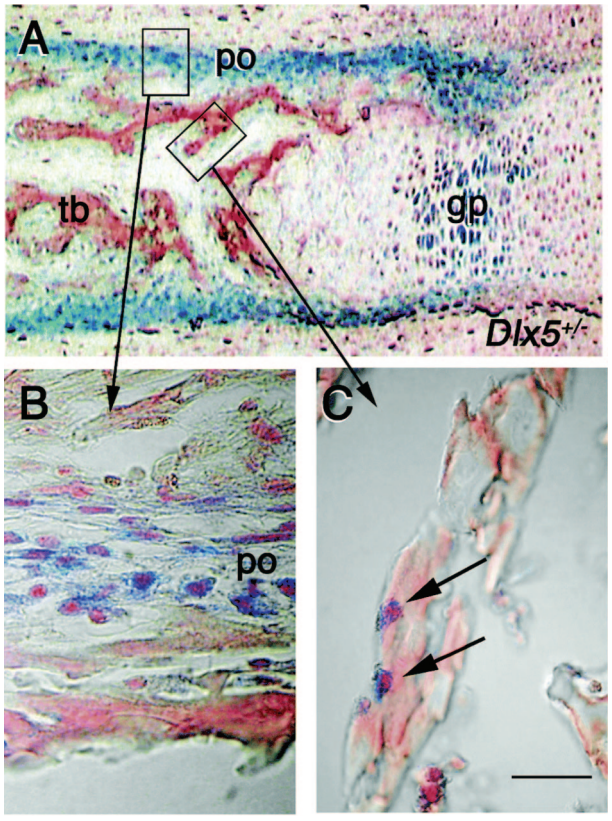

Figure 1. Pattern of β-galactosidase activity on femur sections from E18.5 *Dlx5/lacZ*$^{+/-}$ mice. **A**) Global view of the femur; *LacZ* expression (blue staining) is observed both in the periosteal and in the trabecular region and in growth plate chondrocytes. **B**, **C**) Higher magnification of the periosteal region and of the trabecular region respectively. β-galactosidase activity is seen in most osteoblasts (black **arrows**), but it is strongly reduced in osteocytes. gp, growth plate; po, periosteal region; tb, trabecular bone. Bar: 100 μm in A, 20 μm in B, C.

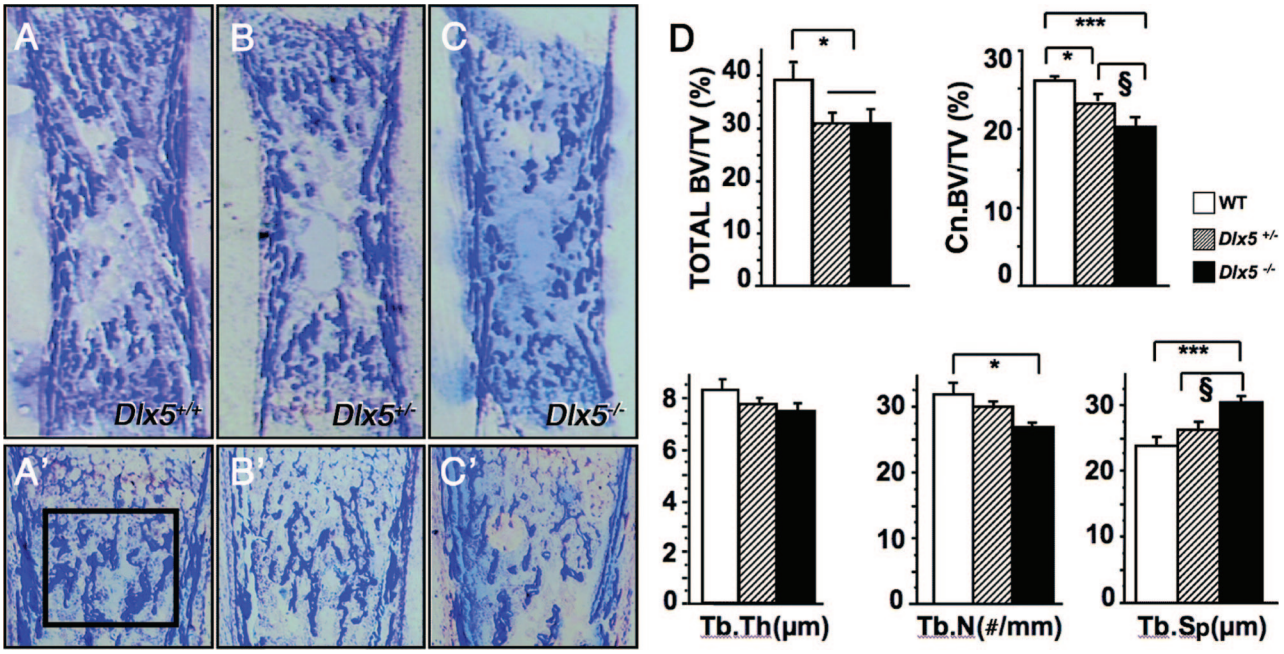

Figure 2. Histomorphometrical analysis. (**A–C**) Undecalcified sections of E18.5 dpc femurs stained with toluidine blue. (**A'-C'**) Primary spongiosa of $Dlx5^{+/+}$, $Dlx5^{+/-}$ and $Dlx5^{-/-}$ femurs; histomorphometric measurements were performed at the trabecular regions indicated by the black box. **(D)** Bar graphs showing the total bone volume (Total BV/TV), cancellous bone volume (Cn.BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular spacing (Tb.Sp) measured for the three genotypes. White bars, $Dlx5^{+/+}$ mice; dashed bars, $Dlx5^{+/-}$ mice and black bars, $Dlx5^{-/-}$ mice. Results are presented as mean ± SEM $^{*}P < 0.05$ and $^{***}P < 0.001$ $Dlx5^{+/-}$ and $Dlx5^{-/-}$ mice relative to wild-type. §, $P < 0.05$ $Dlx5^{-/-}$ relative to $Dlx5^{+/-}$. For total BV/TV measurement $n = 5$ and for the rest $n = 8$ per genotype.

ALP activity in cell lysate was measured at day 7 and 14 using ADVIA®1650 (Bayer Diagnostics, Tarrytown, NY). The activity was normalized to the protein content determined using the BCA protein assay reagent (Pierce Chemical Co, UK). For bone mineralization assay, cells were fixed in 4% paraformaldehyde after 21 days of culture; mineralized nodules were stained by Alizarin Red and counted automatically using the software package Microvision Instruments (Evry, France).

RNA Extraction and Real-Time Quantitative PCR (qPCR)

Purified RNA from calvarial primary osteoblasts cultured for 7 and 14 days was obtained using Nucleospin® (Macherey-Nagel, Easton, PA) and was reverse-transcribed into cDNA using the Reverse-iT Max Blend (ABgene, Surrey, UK). Quantitative real-time PCR expression analysis was performed on Lightcycler1.5 (Roche Diagnostics) using Absolute® SYBR Green capillary mix (ABgene) at 56°C for 40 cycles. Primers product was designed from the online mouse library probes of Roche Diagnostics. mRNA levels were normalized by using either Aldolase A or 18S as housekeeping genes.

In vitro *Osteoclastogenesis*

Spleens from freshly euthanized male adult wild-type mice were used as source of osteoclast precursors. After red blood cell lysis the remaining cells were counted and co-cultured for 8 days in 8-wells Lab-Tek plates (Nunc, Fisher Scientific) with calvaria-derived osteoblasts isolated from 18.5 dpc $Dlx5^{-/-}$ embryo and control littermates (5×10^5 spleen cells, 1×10^4 osteoblasts/well). The culture medium (α-MEM containing 10% FBS) was supplemented with ascorbic acid (50μmol/L) and 10^{-8} mol/L 1,25- dihydroxyvitamin D_3. At the end of the culture period, the wells were trypsinized 5 minutes to detach the osteoblasts cell layers, washed with PBS, fixed with 4% PFA, stained for TRAP and nuclear counterstained with methyl green solution. TRAP-positive multinucleated (number of nuclei >3) cells considered as osteoclast-like cells were counted.

For pit assays 1×10^4 osteoblasts, of each genotype, were seeded on BD BioCoat Osteologic disks followed by 1×10^5 spleen precursors. The culture was maintained 8 days conditions described above. Von Kossa staining was performed according to the manufacturer's instruction. The area of resorption lacunae was quantified on the whole disk using a Nikon microscope interfaced with the Microvision Instruments software package (Evry, France).

Statistical Significance

In vitro experiments were repeated three times independently. Results are expressed as mean ± SE (SEM). Statistical analysis was performed by Statview analysis program using two-way analysis of variance to compare differences between genotypes. *P* values less than 0.05 were considered to be significant.

Results

Dlx5 *is Expressed by Periosteal and Trabecular Osteoblasts*

We have shown that the pattern of β-galactosidase expression in heterozygous *Dlx5*^{lacZ} mice recapitulates faithfully that of *Dlx5* in wild-type mice.[9] In sagittal sections of femurs (Figure 1) of Dlx5$^{+/-}$ mice at E18.5 we observed β-galactosidase activity in growth plate chondrocytes and both in periosteal (Figure 1B) and in trabecular (Figure 1C) osteoblasts while no activity was detected in osteocytes. This observation confirms the known pattern of *Dlx5* expression and suggests a role for this gene in osteoblastogenesis.[9]

Altered Endochondral and Periosteal Bone Development in Dlx5$^{-/-}$ *Mice*

Respiratory lesions cause the perinatal death of *Dlx5*$^{-/-}$ pups.[9] We performed histomorphometric analyses on sections of femurs from E18.5 *Dlx5*$^{-/-}$ embryos and of normal and heterozygous littermates. As at E18.5 cortical bone is not yet completely individualized, to measure the total bone volume we selected the region between the chondro-osseous junctions of femurs (Figure 2A–C). Cancellous bone histomorphometric values were collected on a well-defined area, situated at 100 μm from the growth plate (see Figure 2A'-C'). We observed (Figure 2D) a significant decrease in the total bone volume of *Dlx5*$^{-/-}$ (Total BV/TV −21.4%; $P < 0.05$; $n = 5$) and heterozygous mutants (−21.4%; $P < 0.05$; $n = 5$). Cancellous bone volume was reduced in a gene-dosage dependent fashion (Cn.BV/TV; - 11% and −23% in *Dlx5*$^{+/-}$ and *Dlx5*$^{-/-}$ respectively; $P < 0.001$; $n = 8$). Trabecular number of *Dlx5*$^{-/-}$ mice was reduced (−16%; $P < 0.05$; $n = 8$); conversely, the trabecular separation was increased (+28%; $P < 0.001$; $n = 8$ vs. wild-type embryos; +16%; $P < 0.001$; $n = 8$ vs. *Dlx5*$^{+/-}$) and trabecular thickness was unchanged.

Dlx5 Promotes Osteoblasts Proliferation and Differentiation

We analyzed the effects of *Dlx5* inactivation on the proliferation and differentiation of primary cultures of calvaria-derived osteoblasts. After one to three days in culture the proliferation rate of *Dlx5*$^{-/-}$ and *Dlx5*$^{+/-}$ osteoblasts was about 40% of that displayed by normal cells (Figure 3A) and, although the effect was milder, it was also significantly reduced at later time points. Furthermore, the capacity of *Dlx5*$^{-/-}$ and *Dlx5*$^{+/-}$ osteoblasts to differentiate was similarly hampered as shown by a significant reduction of ALP activity at both 7 and 14 days of culture (Figure 3B) and by the lower number of mineralized nodules formed after 21 days of culture (Figure 3, C and D). By contrast, an allelic dosage effect of *Dlx5* on osteoblast differentiation was observed at 14 days for ALP and 21 days for mineralized nodules formation suggesting its importance for late stage of osteoblast differentiation.

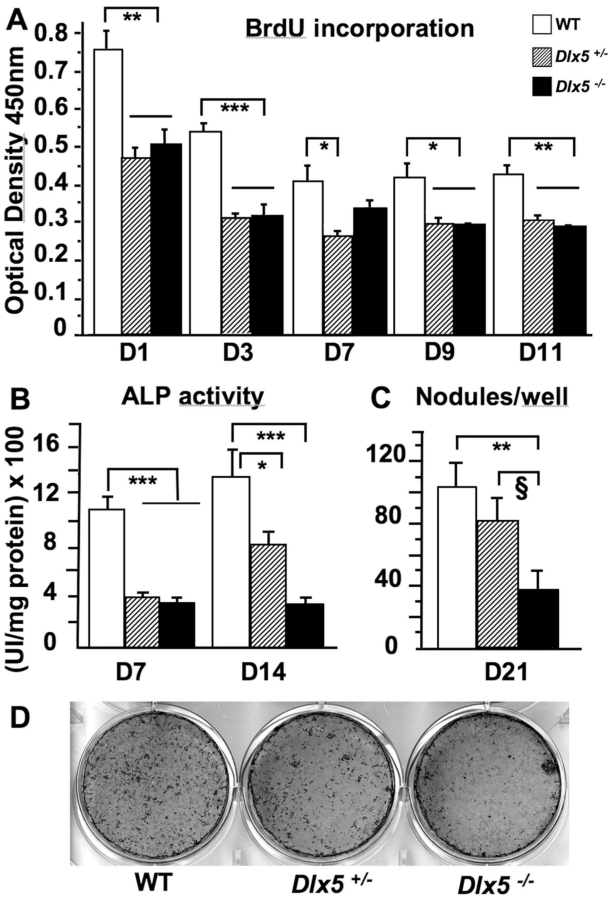

Figure 3. Comparative analysis of cultured *Dlx5*$^{+/+}$, *Dlx5*$^{+/-}$ and *Dlx5*$^{-/-}$ osteoblasts. Osteoblast were isolated from E18.5 dpc wild-type, *Dlx5*$^{+/-}$ and *Dlx5*$^{-/-}$ calvariae. **(A)** Cell proliferation was measured after an 18-hours BrdU pulse administered at different days of culture and expressed as arbitrary units of optical density (OD) at 450 nm. **(B)** Alkaline phosphatase activity measured from osteoblasts cultured for 7 or 14 days in the presence of ascorbic acid; values are normalized to the amount of total protein. **(C)** and **(D)** Number of mineralization nodules obtained after culturing the osteoblasts for 21 days in the presence of ascorbic acid and β-glycerol phosphate. Results are presented as mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *Dlx5*$^{+/-}$ and *Dlx5*$^{-/-}$ mice relative to wild-type. §, $P < 0.05$ *Dlx5*$^{-/-}$ relative to *Dlx5*$^{+/-}$. $N = 4$ wells per genotype at each time point. The result is representative of three independent experiments.

Effect of Dlx5 *Inactivation on the Expression of Bone Developmental* Markers

The expression level of genes involved in the acquisition of the osteoblast phenotype was analyzed in calvaria-derived osteoblasts at 7 and 14 days in culture. As indicated in Figure 4A, the inactivation of both alleles of *Dlx5* led to a significant reduction in the expression levels of *Runx2*, *Osx* and *osteocalcin* at day 7 and 14, and *ALP* at day 14. A direct regulation of *Dlx5* on the BSP gene has been previously suggested, but not yet directly proven in mutant animals.[20] In line with these results, we find also a strong reduction of BSP expression in cultured *Dlx5*$^{-/-}$ and *Dlx5*$^{+/-}$ osteoblasts compared to wild-type osteoblasts at day 7 (Figure 4A). This decreased BSP expression was confirmed *in vivo* by immunohistochemistry (Figure 4B) on decalcified E18.5 wild-type and *Dlx5*$^{-/-}$ femora. These re-

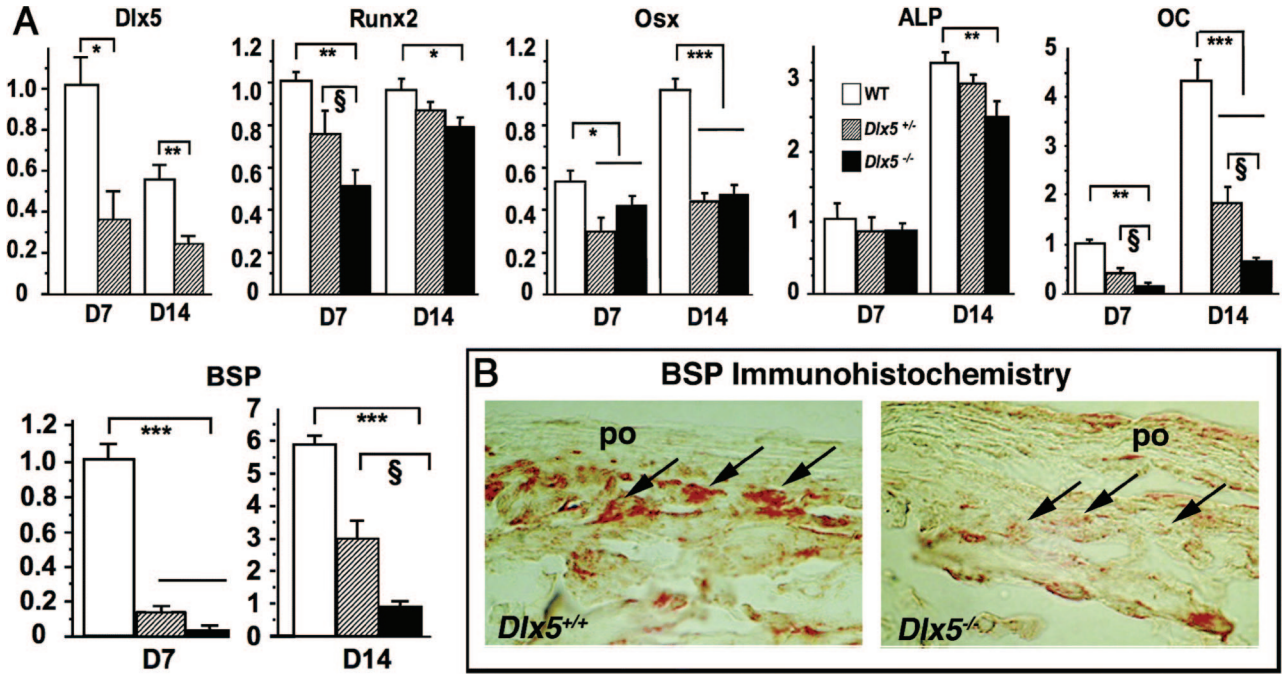

Figure 4. Effects of *Dlx5* inactivation on the expression of bone markers. **(A)** Relative expression levels of *Dlx5*, *Runx2*, *Osterix (Osx)*, *alkaline phosphatase* (*ALP*), *osteocalcin* (*OC*), and *bone sialoprotein* (*BSP*) (determined by real-time PCR) in primary osteoblast cultures at day 7 and day 14. Results are presented as mean ± SEM of triplicates. **(B)** Immunolocalization of BSP protein on sections of wild-type and $Dlx5^{-/-}$ periosteal bone (E18.5 dpc). Black **arrows**: positive cells; po, periosteum. Results are presented as mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ $Dlx5^{+/-}$ and $Dlx5^{-/-}$ mice relative to wild-type. §, $P < 0.05$ $Dlx5^{-/-}$ relative to $Dlx5^{+/-}$. Magnification B: ×1000.

ductions were observed, at a lesser extent, also in hemizygous mutant cells for the late markers of differentiation OC and BSP at day 14.

Dlx5-*Null Mice Have an Increased Resorption Activity*

Bone resorption plays a critical role during bone development and normal bone growth. The decrease in trabecular bone and the increasing trabecular separation observed in *Dlx5*-null mice suggested an overall increase in bone resorption. The number of osteoclasts per bone volume was significantly higher in $Dlx5^{-/-}$ embryos; no significant variation was observed in $Dlx5^{+/-}$ mice (Figure 5A).

A direct effect of Dlx5 on osteoclastic differentiation is unlikely as this gene was not expressed by spleen-derived osteoclast precursors cultured in presence of M-CSF and RANK-L (data not shown). We therefore considered the possibility that *Dlx5* inactivation could affect osteoblast/osteoclast coupling resulting in an increased osteoclast differentiation. We first monitored the levels of expression of OPG and RANKL in cultures of calvaria-derived osteoblasts. After 7 days of culture we observed a significant increase in the RANKL/OPG ratio only in $Dlx5^{-/-}$ osteoblasts suggesting that these cells had a higher potential to induce osteoclastogenesis (Figure 5B).

Next, we co-cultured osteoblasts of the three Dlx5 genotypes with wild-type spleen cells. Osteoclast differentiation was evaluated counting the number of multinucleated TRAP positive cells, and bone resorption activity was determined by measuring the area of resorption pits on calcium phosphate-coated disks after von Kossa staining. After 8 days of co-culture (Figure 5, C and D), we showed that the average number of multinucleated TRAP-positive cells per mm^2 ($Dlx5^{-/-}$: 12.1 ± 1.2 versus $Dlx5^{+/+}$: 5.3 ± 1.3; mean ± SEM), the percentage of resorbed surface ($Dlx5^{-/-}$: 3.71 ± 0.3 versus $Dlx5^{+/+}$: 2.43 ± 0.3%; $n = 4$ wells) and the average size of resorption pits ($Dlx5^{-/-}$: 1534 ± 119 μm^2 versus $Dlx5^{+/+}$: 680 ± 32 μm^2; $n = 4$ wells) were all significantly higher in the co-culture with $Dlx5^{-/-}$ osteoblasts. These results demonstrate that the capacity of *Dlx5*-null osteoblasts to induce osteoclastogenesis is higher than that of wild-type osteoblasts.

Discussion

Bone development, growth and remodelling depend on the tight mutual control of the processes of bone formation and bone resorption mediated respectively by osteoblasts and osteoclasts.[1] Alteration of this balance in favor of osteoclasts leads to excessive bone resorption and to deterioration of bone architecture. Bone homeostasis largely depends on the exchange of signals between osteoblasts and osteoclasts leading to the coupling of the transcriptional regulatory cascades that govern their proliferation and differentiation.[25] Several homeodomain proteins, including members of the Msx and Dlx family play important roles in patterning and formation of skeletal structures during embryogenesis and are supposed to act as upstream regulators of Runx2, a key activator of osteogenesis.[6,12]

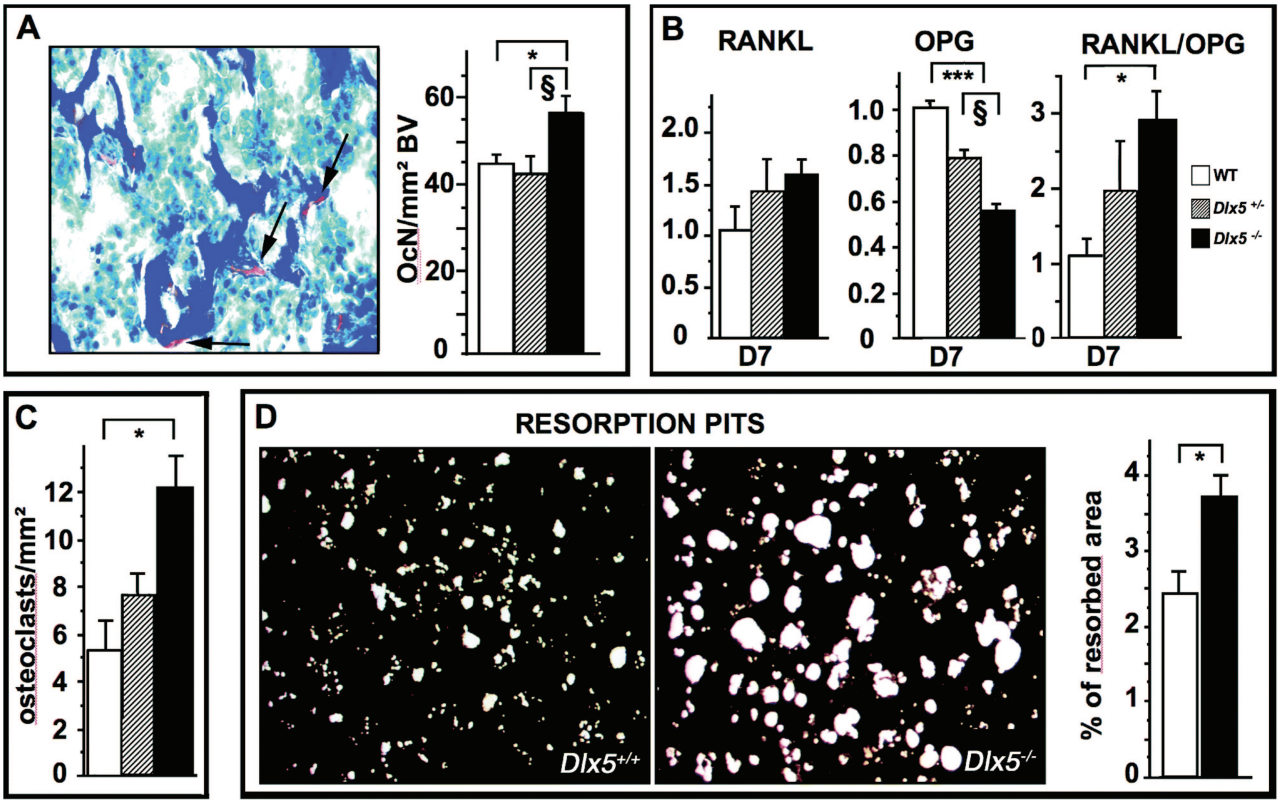

Figure 5. Bone resorption analysis. **(A)** Left: a representative section of the femoral trabecular region stained for TRAP-positive osteoclasts (red cells indicated by **arrows**). Right: number of TRAP-positive cells per mm^2 on sections of *Dlx5*$^{+/+}$, *Dlx5*$^{+/-}$ and *Dlx5*$^{-/-}$ 18.5 dpc femora. **(B)** Relative expression levels of *RANKL* and *OPG* mRNAs (determined by real-time PCR) and RANKL/OPG ratio in primary calvaria osteoblasts cultured for 7 days. **(C)** Number of TRAP-positive multinucleated (number of nuclei >3) cells obtained after co-culturing for 8 days wild-type spleen cells with either wild-type, *Dlx5*$^{+/-}$ or *Dlx5*$^{-/-}$ osteoblasts in the presence of ascorbic acid and 10^{-8} M 1,25- dihydroxyvitamin D_3. **(D)** Left: representative images of the resorption pits obtained on Osteologic disks over which co-cultures of spleen cells and wild-type or *Dlx5*$^{-/-}$ osteoblasts were performed as described in C. Right: quantification of the resorption activity after 8 days of co-culture on Osteologic disks. Results are presented as mean ± SEM for 4 pits. *$P < 0.05$ and ***$P < 0.001$ *Dlx5*$^{+/-}$ and *Dlx5*$^{-/-}$ mice relative to wild-type. §, $P < 0.05$ *Dlx5*$^{-/-}$ relative to *Dlx5*$^{+/-}$.

Dlx5 is expressed by proliferating osteoblasts since very early stages of embryonic bone formation.[7,9] Here we provide direct evidence showing that *Dlx5* promotes osteoblast proliferation and differentiation as indicated by the decreased capacity of *Dlx5*$^{-/-}$cells to express bone differentiation markers and to generate mineralized nodules *in vitro*. Our results extend our knowledge of the hierarchies of transcriptional regulators of bone development.

Runx2 is considered as a master gene for osteoblasts maturation as *Runx2*-deficient mice completely lack bone formation owing to the absence of mature osteoblasts.[11] The osteoblastic defects observed in our study may be in part the result of *Runx2* dependent or independent pathways. Indeed, *Dlx5* has been shown *in vitro* to be a direct transcriptional activator of *Runx2* by binding to its P1 promotor that regulates the transcription of the *Runx2*-II isoform.[14] We confirm these finding *in vivo* showing that *Dlx5* inactivation results in decreased *Runx2* expression. It has been shown[12] that *Dlx5* is competent to promote expression of osteoblast-specific genes such as *ALP* and *osteocalcin* in *Runx2*-null cells suggesting a *Runx2*-independent pathway. The expression of *Osx*, a direct target of Runx2, is also reduced in cultured *Dlx5*$^{-/-}$ osteoblast. This reduction might derive either from an indirect effect of Dlx5 on *Runx2* expression or from a direct action of Dlx5 on *Osx*. Indeed, previous results have shown that *Osx* might be activated through a Dlx5-dependent/Runx2-independent mechanism.[16] The fact that in *Dlx5*$^{-/-}$ mice the process of bone differentiation takes place, suggests that Dlx5 is not as central as Runx2 or Osx, but only acts as a modulator of their expressions. Osteocalcin and BSP, both markers of differentiated osteoblasts implicated in the process of mineralization, have been reported to be under the direct transcriptional control of Dlx5.[19,26] We confirm these findings as both markers are drastically down-regulated in *Dlx5*$^{-/-}$ osteoblasts in culture. Our study shows, therefore, that *Dlx5* is not only an activator of osteoblast proliferation and early differentiation but can affect also later stages of osteogenesis.

We show that the inactivation of a single *Dlx5* allele results in lower levels of its expression by osteoblasts. Interestingly, certain parameters of bone development (Cn.BV/TV and Tb.Sp) and the levels of expression of *Runx2, OC* and *BSP* change gradually in response to the allelic dosage of *Dlx5* while others (Total BV/TV, proliferation rate) and *osterix* expression show the same reduction in homozygous and heterozygous mutant mice. These data suggest that Dlx5 regulates downstream genes in a gene-dosage dependent fashion and that in certain cases a threshold effect in gene regulation might also be present.

Taken together our *in vitro* results correlate well with the remarkable reduction of total bone volume observed *in vivo*. This correlation is, however, not seen for trabecular bone as no significant difference in trabecular thickness, an indicative parameter of osteoblastic activity, is observed in *Dlx5*$^{-/-}$ mice. The different origin of cortical and trabecular osteoblasts precursors may partly explain this different phenotype. Indeed cultured precursors derive from the calvaria and their differentiation may be better correlated to the formation of the cortical component of long bones as they both are of intramembranous origin.

In addition to impairment of calvaria-derived osteoblast function, *Dlx5*$^{-/-}$ mice exhibited a significant increase in osteoclast number and trabecular separation as shown by our histomorphometric measurements. *Dlx5* is not expressed by differentiated multinucleated TRAP positive osteoclasts; we focused therefore our attention on the cross talk between osteoblasts/osteoclasts and in particular on the molecular triad OPG/RANK/RANKL, which orchestrates osteoclastogenesis and bone resorption.[25] Our findings show that *Dlx5*$^{-/-}$ osteoblasts indirectly enhance osteoclastogenesis. Mutant osteoblasts present an increased RANKL/OPG ratio supporting the notion of an imbalanced osteoblasts/osteoclasts coupling. The increased number of multinuclear TRAP positive cells obtained in *Dlx5*$^{-/-}$ co-cultures is actually associated with a higher resorption activity indicating a higher number of functional osteoclasts. The increased size of the resorption pits observed in *Dlx5*$^{-/-}$ co-cultures might either result from an increase in the number of osteoclasts or from an increased resorption activity of individual cells. The fact that *Dlx5*$^{-/-}$ osteoblasts are relatively undifferentiated might favor osteoclastogenesis as observed in several studies where the differentiation status of osteoblasts affected osteoclast formation and function.[27] As in *Runx2*$^{-/-}$ mice the osteoclast number and the RANK-L/OPG ratio are drastically decreased, it appears that the action of Dlx5 on osteoclastogenesis is independent of Runx2 regulation.[28]

To our knowledge no other mutant mouse model displays a phenotype of osteoblast/osteoclast coupling associated to an increased resorption activity similar to that observed in *Dlx5*$^{-/-}$ mutants. Our findings might lead to the development of new tools to understand the origin of bone homeostasis-related diseases such as osteoporosis or osteopenia resulting from immobilization as it has been shown that *Dlx5* expression increases in the presence of mechanical loading thus altering the coupling between osteoblasts and osteoclasts.[29,30]

The analysis of other genetically modified models allowing the study of bone remodelling, such as mice carrying a conditional invalidation of *Dlx5* in bone, will be decisive to evaluate the potential implication of *Dlx5* in osteoporosis.

Acknowledgments

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